Cd-MT causes endocytosis of brush-border transporters in rat renal proximal tubules

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Nephrotoxicity in humans and experimental animals due to chronic exposure to cadmium (Cd) is manifested by defects in the reabsorptive and secretory functions of proximal tubules (PT). The main symptoms of Cd nephrotoxicity, including polyuria, phosphaturia, aminoaciduria, glucosuria, and proteinuria, suggest that various brush-border membrane (BBM) transporters are the main targets of Cd. Specific transporters may be either directly inhibited by Cd or lost from the BBM after Cd treatment, or both. We have recently proposed that Cd may impair the vesicle-dependent recycling of BBM transporters by inhibiting vacuolar H⁺-ATPase (V-ATPase) activity and endocytosis in PT cells (Herak-Kramberger CM, Sabolic I, and Brown D. Kidney Int 53: 1713–1726, 1998). The mechanism underlying the Cd effect was further explored in an in vivo model of experimental Cd nephrotoxicity induced by Cd-metallotriopein (Cd-MT; 0.4 mg Cd/kg body mass; a single dose sc) in rats. The inhibition in vitro may result from the loss of BBM functional capacity due to 1) shortening and loss of microvilli, 2) time-dependent loss of megalin, V-ATPase, aquaporin-1 (AQP1), and type 3 Na⁺/H⁺ exchanger (NHE3) from the BBM, 3) redistribution of these transporters into vesicles that were randomly scattered throughout the cell cytoplasm, and 4) redistribution of NHE3, not but megalin, into the basolateral plasma membrane. The internalization of BBM transporters was accompanied by fragmentation and loss of microtubules and by an increased abundance of α-tubulin monomers in PT cells. Transporter redistribution was detectable as early as 1 h after Cd-MT treatment and increased in magnitude over the next 12 h. We conclude that the early mechanism of Cd toxicity in PT cells may include a colchicine-like depolymerization of microtubules and impaired vesicle-dependent recycling of various BBM proteins. These processes may lead to a time-dependent loss of cell membrane components, resulting in reabsorptive and secretory defects that occur in Cd-induced nephrotoxicity.

Nephropathy in humans and experimental animals due to chronic exposure to cadmium (Cd) is manifested by reabsorptive and secretory dysfunction of renal tubules. The principal symptoms of tubular damage (proteinuria, phosphaturia, glucosuria, aminoaciduria, and hyperosmolar polyuria) indicate that Cd preferentially targets various transporters in the proximal tubule brush-border membrane (BBM) (3, 5, 30, 34, 35, 37). Several Na⁺-dependent BBM transporters, such as those for phosphate (NaPi-2a) (4, 5, 30), glucose (37), and amino acids (34), were inhibited by inorganic Cd in isolated BBM vesicles and in experimental animals treated with CdCl₂ for 2–3 wk. The inhibition in vitro may be due to a direct interaction of Cd with the transporters (4, 5, 34, 36, 37, 58) and/or a loss of BBM vesicle integrity (29). However, tubular dysfunction in vivo may result from the loss of BBM functional capacity due to 1) shortening and loss of microvilli (19, 28), 2) loss of specific BBM transporters (28, 30), 3) direct inhibition of BBM transporters by Cd (4, 5, 28, 34, 36, 37, 50, 58); 4) indirect inhibition of BBM transporters caused by oxidative stress and peroxidation of membrane lipids (60); and 5) a combination of these phenomena.

How Cd reaches and damages proximal tubule cells is not entirely clear. While acute exposure of experimental animals to a high dose of CdCl₂ has little nephrotoxic effect, nephrotoxicity develops with either repeated parenteral small daily doses of CdCl₂ or after prolonged oral ingestion of this substance. In the liver, either treatment may stimulate the synthesis of a cystein-rich metal-binding protein, metallothionein (MT), to which Cd binds and forms a Cd-MT complex (6–7 kDa) that may be released into the circulation (21). However, Cd-MT in the blood may also originate from food, after absorption of an intact molecule in the gastrointestinal tract (15, 16) or after binding of in-

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gested inorganic Cd to MT in gastrointestinal tract cells and release of this complex into the circulation (33). The circulating Cd-MT may be filtered by the glomeruli, endocytosed in proximal tubule cells, and degraded in lysosomes (20). The released Cd may then stimulate production of MT in proximal tubule cells (23, 59) and target various cellular structures and functions (28, 30, 31, 41, 55, 60, 67).

The possibility that Cd-MT mediates Cd action in the kidney has been used to develop short-term animal models of Cd nephrotoxicity. Animals treated with a single dose of Cd-MT developed full-blown structural and functional damage in Cd-MT (16, 22, 61, 62) and functional (22, 32, 63, 64) damage required weeks or months to manifest. The damage induced by a single or, in some studies, repeated Cd-MT injections qualitatively resembled those achieved by long-term treatment with CdCl2 (23, 38, 47, 70), although, for unknown reasons, they occurred at much lower levels of tissue Cd than after treatment with inorganic Cd (21, 31, 48).

The intracellular events in proximal tubule cells that lead to structural and functional damage in Cd-MT nephrotoxicity have not been established. In proximal tubule cells of rats treated with CdCl2 for 2 wk we recently showed (1) greatly diminished expression of NaPi-2a and the vacular H+ -ATPase (V-ATPase), 2 impaired endocytosis of a fluorescent marker, FITC-dextran, and 3) deranged and partially depolymerized actin and microtubule cytoskeleton (28, 30, 55). Because V-ATPase-mediated acidification of intracellular organelles (42) and the cytoskeleton (6, 11, 14) play a pivotal role in the trafficking, targeting, and recycling of many plasma membrane proteins, we assumed that Cd may acutely interfere with these pathways and thereby affect the structural and functional polarity of proximal tubule cells. The experiments in this report were designed to examine the early intracellular effects of Cd-MT in proximal tubules that may contribute to the development of Cd nephrotoxicity.

MATERIALS AND METHODS

Animals and treatment. Two-month-old male Wistar rats (body mass, 180–200 g) from the breeding colony at the Institute in Zagreb were used. Animals were bred and maintained according to the Guide for Care and Use of Laboratory Animals (Washington, DC: Academy Press, 1996). Before and during experiments, animals had free access to standard laboratory food and tap water. The studies were approved by the Institutional Ethics Committee.

Cd-MT (from rabbit liver, 7% metal as Cd; Sigma, St. Louis, MO) was dissolved in 0.9% NaCl and injected subcutaneously in a single dose of 0.4 mg Cd/kg body mass. The animals were killed at various times (hours) thereafter. As shown previously (32), 8–16 h later, the above-mentioned treatment induced urinary symptoms of nephrotoxicity that included proteinuria and calciuria. Control animals received an equivalent amount (0.2–0.3 ml) of vehicle 12 h before death. In preliminary experiments, by checking the tissue content of Cd and the immunocytochemical distribution of various antigens, we showed that these parameters in untreated rats and in rats treated with vehicle 6 or 12 h before death were similar (data not shown). In one experiment, to compare effects of inorganic Cd, rats were injected subcutaneously with a solution of CdCl2 (in water) in a single dose of 0.4 mg Cd/kg body mass, and the animals were killed at various time points (hours) thereafter.

Determination of tissue cadmium. Rats were decapitated at various time points (hours) after treatment with Cd-MT, and the kidney cortex was weighed and dry ashed at 450°C for 24 h. Ashed samples were dissolved in 2% nitric acid, and the Cd concentration was measured by atomic absorption spectrometry (Varian-AA375; flame mode) using an appropriate standard solution (999 ± 2 mg Cd/l; 19777.0500, Merck, Darmstadt, Germany) as a reference.

Antibodies. Primary antibodies included polyclonal (rabbit immune serum) and monoclonal (1H2) anti-megalin holoprotein antibodies (1, 6, 26); polyclonal antibodies (whole rabbit immune serum and an anti-rabbit immune serum or an antibody purified by immunoadsorption onto Immobilon strips containing AQPI protein (57); and monoclonal antibodies (cell culture media) 19F5 and 2B9 against a type 3 NHE3 (57). A commercial monoclonal antibody (affinity purified to α-tubulin (T-9026, Sigma) was used to label microtubules (6). The reason for the use of some polyclonal antibodies in the form of either immune serum or affinity-purified IgG was indicated in our previous publication (27): whereas the specificity of labeling with both forms was the same, the immune sera and the affinity-purified antibodies gave stronger labeling in immunoblotting and immunocytochemical studies, respectively.

Secondary antibodies were purchased commercially from either Jackson ImmunoResearch, West Grove, PA (fluorescein-labeled antibodies) or Kirkegaard and Perry, Gaithersburg, MD (alkaline phosphatase-labeled antibodies) and included fluorescein- or alkaline phosphatase-labeled goat anti rabbit IgG (GARF or GARAP, respectively); fluorescein-labeled donkey anti-chicken IgG (DACF); and fluorescein- (GAMF) or alkaline phosphatase-labeled goat anti-mouse IgG (GAMAP).

Tissue fixation and immunocytochemistry. Rats were anesthesized with Nembutal (65 mg/kg body mass ip) and then perfused via the left cardiac ventricle, first with PBS [(in mM) 140 NaCl, 4 KCl, 2 KH2PO4, pH 7.4] at 37°C for 2–3 min to remove circulating blood and then with 180 ml PLP fixative (2% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate) for 5 min (43). Kidneys were removed, decapsulated, sagitally sliced, and kept overnight in the same fixative at 4°C, followed by extensive washing in PBS and storage in PBS containing 0.02% NaN3 at 4°C until further use.

To cut 4-μm frozen sections, tissue slices were infiltrated with 30% sucrose (in PBS) overnight, frozen in liquid nitrogen, and sectioned in a Leica CM 1850 cryostat (Leica Instruments, Nussloch, Germany). Sections were collected on Superfrost/Plus Microscope slides (Fisher Scientific, Pittsburgh, PA) and rehydrated in PBS for 10 min. Sections for V-ATPase and NHE3 staining were pretreated for 5 min with 1% SDS (in PBS) to expose cryptic antigenic sites (12); SDS was removed by extensive washing with PBS. Sections for megalin, AQPI, and tubulin staining were used without SDS pretreatment. Nonspecific binding of antibodies was reduced by incubating sections with 1% bovine serum albumin (in PBS) for 15 min before application of antibodies against...
megalin (immune serum diluted 1:800 with PBS); V-ATPase (affinity-purified chicken antibody, 1:20); AQP1 (affinity purified, used undiluted); NHE3 (cell culture medium 19F5, 1:20); and α-tubulin (affinity-purified antibody, 1:50) in a refrigerator overnight (for 12–14 h). This was followed by two washes with high-salt PBS (PBS containing 2.7% NaCl) to decrease the nonspecific binding of antibodies, plus two washes in regular PBS (5 min each). The sections were then incubated with the respective secondary antibodies GARF or GAMF (8 μg/ml in PBS) for 1 h, washed twice with high-salt PBS and twice with regular PBS (5 min each), mounted in a fluorescence-fading retardant (Vectashield; Vector Laboratories, Burlingame, CA), and examined and photographed with a camera-equipped Opton fluorescence microscope (Jena, Germany) using Kodak TMAX 400 or Kodak Elite slide films push-processed to 800 ASA.

Immunogold electron microscopy. The immunogold-labeling of ultrathin frozen tissue sections was performed according to the method of Tokuyasu (65). The kidney cortex was infiltrated with 2.3 M sucrose overnight, frozen in liquid nitrogen, and 70- to 80-nm-thick sections were cut on a Leica ultracryomicrotome (EMFC Ultracut UCT) and mounted on Formvar-coated nickel grids. The grids with sections were processed at room temperature in a humid chamber using the following steps: rehydration with PBS containing 20 mM glycine for 10 min followed by PBS alone (2 × 5 min); blocking with 1% BSA in PBS (BSA/PBS) for 15 min; incubation with 1H2 (1:500) or anti-NHE3 antibody (19F5; 1:20) for 30 min; washing with BSA/PBS for 5 min and PBS alone (3 × 5 min); incubation with gold-conjugated goat anti-mouse IgG antibody (10-nm gold particles, Electron Microscopy Sciences, Fort Washington, PA; diluted 1:5) for 60 min; washing in 2.7% NaCl (PBS containing 2.7% NaCl) to washes with high-salt PBS (PBS containing 2.7% NaCl) to precipitate the secondary antibodies. The grids were stained for alkaline phosphatase activity with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium method. The density of specific protein bands was scanned (Ultrascan XL Laser Densitometer, LKB, Bromma, Sweden), and the integrated surface of each scan was expressed in arbitrary units relative to the surface of the densest band = 100 arbitrary units) in samples from control animals.

Presentation of the data. The immunofluorescence and immunogold-labeling data represent findings from three rats in the control and each experimental group. The figures were prepared from color slides or black-and-white negatives that were scanned using a Polaroid SprintScan 35 Plus scanner or an Epson Perfection 1640 SU with an Epson EU-33 transparency adaptor. Scans were imported into Adobe Photoshop 4.0 software, appropriately processed and labeled, and printed on an Epson Stylus Color printer. The numerical data (means ± SE) were statistically evaluated either by two-tailed t-test or by ANOVA/MANOVA, followed by Dunnett's multiple-range test at 5% level of difference. The evaluation was performed using Statistica 5.0 for Windows (release 1995; www Statssoft.com).

RESULTS

Effect of Cd-MT treatment on Cd content of renal cortical tissue. Cd concentration in wet and dry renal cortex was determined in control rats (injected with saline 12 h before death; time 0) and in rats injected with Cd-MT and killed 3, 6, or 12 h later. As shown in Table 1, 3 h after Cd-MT injection, the wet tissue Cd concentration reached 40 μg/g; i.e., it increased 1,000-fold compared with the amount in control animals. The accumulated Cd remained unchanged at 6 h and slightly decreased (12%) at 12 h after Cd-MT injection. However, the dry tissue Cd, which increased 1,067-fold 3 h after Cd-MT injection, increased further by 37% (1,462-fold compared with that in the controls) at 6 h, indicating an increased abundance of tissue water (edema) at this time point. At 12 h after Cd-MT treatment, the dry tissue Cd was 30% lower than at 6 h. Thus the injection protocol used in these experiments.
was successful in raising the tissue Cd levels considerably, as reported previously (16).

Distribution of BBM transporters in proximal tubules after Cd-MT treatment. Megalin, an apical membrane protein in proximal tubule cells, is a receptor involved in the reabsorption of several filtered proteins (18). Treatment of rats with Cd-MT induced in most (but not in all) tubules a major, time-dependent intracellular redistribution of megalin (Fig. 1, A–H). In cells of proximal convoluted tubules in control animals, in accordance with previous observations (6, 14, 26), the anti-megalin antibody strongly stained the BBM and apical membrane coated pits, with little staining of the rest of the cytoplasm (Fig. 1A). Already at 1 h after Cd-MT treatment, the apical staining became weaker and thinner in most tubules, and numerous granulélike protrusions of staining (which we have termed “granulation”) were observed projecting from the apex of the cell into the cytoplasm (Fig. 1, B and C). Two hours after the injection of Cd-MT, the intensity of the apical staining was further diminished, and numerous small intracellular vesicles were seen in the subapical domain of many cells (Fig. 1D). This process continued with time, resulting in progressively decreased apical staining and an increased abundance of labeled intracellular vesicles 3 (Fig. 1E), 4 (Fig. 1F), 6 (Fig. 1G), and 12 h (Fig. 1H) after Cd-MT injection. In addition to this cytoplasmic accumulation of megalin, many tubules exhibited various signs of necrosis 6 and 12 h after Cd-MT treatment, including cell edema, pycnotic nuclei, damaged apical surface, loss of epithelium, and presence of cell debris in the tubule lumen. This typical structural damage for Cd nephrotoxicity is clearly seen in Fig. 1H and was previously observed in animals treated with Cd-MT (16, 22, 61, 62) or subchronically intoxicated with CdCl₂ (19, 23, 28).

The Cd-MT-induced redistribution of apical proteins in proximal tubule cells was not restricted to megalin. V-ATPase was located in the apical domain in control rats (Fig. 2A), whereas 6 h after Cd-MT treatment the antigen was redistributed intracellularly (Fig. 2B). AQPI staining in control animals was localized to the BBM and basolateral membrane (BLM) (Fig. 2C); 6 h after Cd-MT treatment, the staining intensity in both membrane domains was diminished in many (but not in all) tubules, and AQPI was relocated into cytoplasmic vesicles (Fig. 2D). Also, apical NHE3 staining in tubules from control rats (Fig. 2E) was diminished in the apical domain in many tubules and was redistributed into intracellular vesicles 6 h after Cd-MT injection (Fig. 2F). However, the overall loss of NHE3 from the BBM and its intracellular relocation tended to be less extensive than that observed for megalin, V-ATPase, and AQPI. In addition, in some tubules we also observed a faint basolateral NHE3 staining, which in Fig. 2F was largely masked by the intensive background red color of Evan’s blue; this basolateral staining was stronger 12 h after Cd-MT treatment (see Relocation of BBM transporters into the BLM: immuno-gold labeling).

As mentioned above, all the proximal tubules were not equally affected by Cd-MT; even 12 h after Cd-MT treatment, a number of tubule profiles showed only a weak internalization or even normal distribution of megalin and other antigens (data not shown). Furthermore, none of the antigens examined was affected in the proximal tubule S3 segment even 12 h after Cd-MT injection (data not shown), thus confirming previous observations in which treatment of rats with Cd-MT or CdCl₂ did not affect the structure and function of this segment (16, 20, 22). Furthermore, in one experiment, Cd-MT (0.4 mg Cd/kg body mass) was injected into the

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<td>Tissue Cd, µg/g</td>
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<td>Wet mass</td>
<td>0.04 ± 0.01 †</td>
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<td>Dry mass</td>
<td>0.21 ± 0.07 †</td>
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<td>Yield of BBM, mg BBM protein/g tissue</td>
<td>6.39 ± 0.32 a</td>
<td>5.36 ± 0.23 b</td>
<td>4.68 ± 0.39 bc</td>
<td>4.24 ± 0.20 c</td>
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<td>Density of specific protein bands (arbitrary units)</td>
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<td>Megalin (31-kDa subunit)</td>
<td>99 ± 3.8 a</td>
<td>88 ± 1.9 a</td>
<td>69 ± 6.4 b</td>
<td>67 ± 2.8 b</td>
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<td>V-ATPase</td>
<td>94 ± 3.0 a</td>
<td>69 ± 2.4 b</td>
<td>48 ± 6.2 c</td>
<td>41 ± 6.3 c</td>
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<tr>
<td>NHE3</td>
<td>95 ± 2.1 b</td>
<td>98 ± 6.6 c</td>
<td>94 ± 7.4</td>
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<td>-Tubulin</td>
<td>86 ± 8.5 a</td>
<td>100 ± 20.2 a</td>
<td>60 ± 7.6</td>
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<td>AQP1</td>
<td>82 ± 12.3 †</td>
<td>149 ± 24.8 b</td>
<td>172 ± 11.7 b</td>
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Values are means ± SE. Cd-MT, cadmium-metallothionein; BBM, brush-border membrane; AQP1, aquaporin-1; NHE3, type 3 Na⁺/H⁺ exchange. Cd in wet and dry tissues and protein in BBM preparations were determined in samples from 3 (12 h) or 4 (0, 3, and 6 h) animals. Band densities were determined in 3 (megalin), 6 (NHE3), or 7 vacuolar (V-ATPase, AQP1) BBM preparations or in 4 tissue homogenates (α-tubulin) in each group. Densitometric data for AQP1 are shown only for the nonglycosylated form (28 kDa) of the protein. (P < 0.05; ANOVA/Duncan’s multiple-range test) are found by comparing the data indexed with b vs. a and c vs. a or b. Data indexed with the same letters and nonindexed data showed no statistical difference in any combination.
jugular vein (iv) instead of subcutaneously. The redistribution of antigens examined in proximal convoluted tubules exhibited exactly the same pattern of time-dependent redistribution after intravenous and subcutaneous treatment (data not shown). Finally, a group of rats was injected subcutaneously with an equivalent, single dose of CdCl2 (0.4 mg Cd/kg body mass) instead of Cd-MT to test the specificity of Cd-MT action; although 6–12 h later most tubules exhibited signs of initial granulation with the anti-megalin antibody, further internalization of this apical antigen, as observed after Cd-MT injection, did not occur (data not shown).

Relocation of BBM transporters into the BLM: immunogold labeling. The basolateral localization of some apical transporters in Cd-MT-treated rats, e.g., NHE3, was studied in more detail in rats that had been treated with Cd-MT 12 h earlier, e.g., when the redistribution of megalin, V-ATPase, AQP1, and NHE3, in proximal tubules was extensive (Fig. 3). In the case of megalin, the basolateral domain of most tubules was unstained, but some tubules exhibited a weak staining at their basolateral pole (Fig. 3A). This basolateral megalin may be confined to intracellular vesicles located in the vicinity of the BLM, as previously described in colchicine-treated rats (26). The V-ATPase was also strongly internalized, but the basolateral pole of the proximal tubule remained unstained (Fig. 3B). On the other hand, in many tubules with extensive endocytosis of apical AQP1 still exhibited a substantial basolateral staining of this antigen.
In contrast, a clear and sharp staining of NHE3 in the BLM was always found in proximal tubules after 12 h of Cd-MT treatment, in parallel with a reduced intensity of apical staining (Fig. 3D). To examine the abundance of BBM antigens before and after Cd-MT treatment, and to test whether the basolateral megalin and NHE3 staining observed in the preceding experiment was indeed located in the BLM, we compared immunogold-labeling patterns in ultrathin frozen tissue sections using anti-megalin and anti-NHE3 antibodies (Figs. 4 and 5, respectively). In control kidney cortex, 1H2 markedly labeled the external surface of brush-border microvilli and the internal surface of subapical vesicles in the proximal convoluted tubule (Fig. 4A). No significant gold labeling was observed in the BLM of these cells (Fig. 4C). In rats treated for 12 h with Cd-MT, microvilli in the affected cells were generally shorter, irregular in height (Fig. 4B), and focally lost in many places (not shown). The number of gold particles over the microvilli (particles/μm length of membrane) was measured in 8–10 randomly chosen microvilli in representative cells from these rats (n = 3). The labeling found in control rats (3.92 ± 1.01; n = 3) was strongly decreased (0.19 ± 0.19; n = 3; t-test: P < 0.05) after 12-h exposure to Cd-MT. However, gold particles were abundant in many intracellular vesicles (Fig. 4B). At the basolateral domain of these cells, the gold particles decorated the internal surface of numerous vesicles positioned in the vicinity of the BLM, but the BLM itself was unlabeled (Fig. 4D).

With use of the same technique, NHE3 antibodies heavily labeled the external surface of brush-border microvilli (12.2 ± 0.67 particles/μm; n = 3) and the internal surface of subapical vesicles in proximal tubule cells from control rats (Fig. 5A). No significant labeling was observed in the BLM of these cells (Fig. 5C). In morphologically damaged microvilli of Cd-MT-
treated rats, the number of particles per micrometer actually increased 54% over the control level (18.8 ± 1.95 particles/μm; n = 3; t-test: P < 0.05), and numerous particles were visible in many intracellular vesicles (Fig. 5B). At the basolateral domain of these cells, the internal side of intracellular vesicles and the external side of the BLM were heavily labeled (Fig. 5D).

Immunoblotting of isolated BBM. Renal cortical BBM from control and Cd-MT-treated rats were immunoblotted with the same battery of antibodies used for immunocytochemical studies. The final membrane preparations from all animal groups were similarly enriched in LAP activity [enrichment factor was 9.6 ± 0.83 (n = 4), 10.2 ± 0.83 (n = 4), 10.5 ± 0.89 (n = 4), and 11.9 ± 0.35 (n = 3) in BBM from control rats and from rats treated with Cd-MT 3, 6, and 12 h earlier, respectively] and were also contaminated to a similar extent with thrombomodulin and Na⁺-K⁺-ATPase, markers for endothelial cell plasma membranes (52) and proximal tubule cell BLM, respectively (data not shown), indicating that the membranes isolated from the different groups of rats were similar in origin. However, the yield of BBM, expressed as milligrams of BBM protein obtained per gram of cortical tissue, showed a steady decline, being 84, 73, and 66% of that in control rats 3, 6, and 12 h after Cd-MT treatment, respectively (Table 1).

Representative immunoblots, obtained with two independent BBM preparations from each animal group, are shown in Fig. 6, and the densitometric values of the specific protein bands, pooled from two separate experiments, are listed in Table 1. The abundance of megalin (molecular mass: ~520 kDa) and the 31-kDa V-ATPase subunit in isolated BBM steadily diminished 3, 6, and 12 h after Cd-MT treatment (Fig. 6, A and B, respectively; Table 1). This finding was in accordance with the time-dependent reduction in apical immunofluorescence observed with the respective antibodies and its redistribution into intracellular vesicles. However, the abundance of AQP1 and NHE3 in isolated BBM exhibited no consistent decline (Fig. 6, C and D, respectively; Table 1), which is in apparent contrast to the

Fig. 3. Immunolocalization of megalin (A), V-ATPase (B), AQP1 (C), and NHE3 (D) in proximal convoluted tubules of rats treated with Cd-MT 12 h earlier. The redistribution of megalin was largely intracellular in most tubules; in some tubules, however, a limited staining at the basolateral pole of the cells was also observed (A, arrows). V-ATPase was also redistributed into intracellular vesicles; the basolateral pole of the cells remained unstained (B). In the tubules with a marked internalization of AQP1 (y), both apical and basolateral cell membranes were weakly stained. In contrast, in tubules that showed less internalization of the antigen (x), significant AQP1 staining was still detectable in both membrane domains (C). In most tubules with a marked redistribution of NHE3, staining was localized to intracellular vesicles and the basolateral membrane (BLM; D, arrows). Bar = 20 μm.
marked reduction in these antigens that was found by immunofluorescence microscopy (cf. Fig. 2, C and D and E–F, respectively). However, the quantitative immunogold-labeling data described above show that the amount of NHE3 gold label per micrometer length of BBM is not decreased after Cd-MT treatment and even appears greater than in control tissues. Thus the observed decrease in apical NHE3 staining detected by immunofluorescence microscopy (Fig. 2F) is due to a general reduction in size and number of the brush-border microvilli and not to a decrease in concentration of the protein in the apical plasma membrane after Cd-MT treatment. In contrast, the concentration of megalin in the apical membrane does decrease after Cd-MT treatment, resulting in a parallel reduction in both immunocytochemical staining and immunoblotting.

Microtubules. The time-dependent internalization of various apical transporters and the finding of NHE3 in the BLM in proximal tubules of Cd-MT-injected rats described above is remarkably similar to the effect on these transporters of the microtubule-depolymerizing agent colchicine in rats (6, 13, 26, 56). Therefore, the effect of Cd-MT treatment on microtubule organization in proximal tubules was examined. In accordance with previous findings from our laboratory (2, 6, 56), apical-to-basolateral bundles of microtubules were abundant in proximal tubule cells of control rats (Fig. 7A). However, already 1 h after Cd-MT injection there was a substantial loss of overall staining intensity as well as a shortening and fragmentation of microtubules in most cortical proximal tubules (Fig. 7B). The loss of staining was more marked after 2 h (Fig. 7C) and was nearly complete 3–4 h after Cd-MT injection (Fig. 7D). At 6 and 12 h, a partial reestablishment of the microtubule network was apparent in some cells,

Fig. 4. Immunogold labeling of megalin in the apical (A and B) and basolateral (C and D) domains of proximal tubule cells in rats treated with saline (controls; A and C) or Cd-MT 12 h earlier (B and D). In control animals, labeling was present at the external side of the brush-border membrane (BBM; arrowheads) and at the internal side of sub-apical vesicles (A, arrow); the labeling tended to be stronger at the base of microvilli. The BLM of the same cells was not significantly labeled (C). In Cd-MT-treated rats, microvilli were shorter, irregular in height, and almost devoid of gold particles, whereas the labeling was abundant in intracellular vesicles (B, asterisks). At the basolateral pole of the same cells, the internal side of numerous intracellular vesicles in the vicinity of BLM was labeled (asterisks), but the BLM remained unlabeled (D, arrowheads). Bar = 0.5 μm.
albeit in a somewhat abnormal pattern (Fig. 7, E and F, respectively). However, despite the loss of polymerized microtubules, the abundance of α-tubulin in cortical homogenates from Cd-MT-treated rats increased 82, 110, and 198% above control values, 3, 6, and 12 h after Cd-MT injection, respectively (Fig. 6E, Table 1).

**DISCUSSION**

To gain greater insight into the mechanism of Cd nephrotoxicity in mammals, the acute effects of Cd-MT treatment in rats on the distribution of several BBM transporters and microtubules in proximal tubules were studied. In proximal tubule cells of Cd-MT-treated rats, we observed 1) shortening and loss of microvilli; 2) time-dependent loss of megalin, V-ATPase, AQPI, and NHE3 from the BBM; 3) redistribution of these transporters into randomly scattered cytoplasmic vesicles; 4) redistribution of NHE3, but not megalin, into the BLM; and 5) time-dependent fragmentation and loss of microtubules, accompanied by an increased abundance of α-tubulin monomers.

The major finding was that Cd-MT induced a rapid, time-dependent internalization of several apical membrane transporters. The transporters initially accumulated in subapical invaginations (granulation pattern), followed by their continued loss (by endocytosis) from the BBM and relocation into vesicles that were randomly scattered throughout the cytoplasm. This process was already apparent 1 h after Cd-MT treatment and increased in magnitude over the next 12 h. The time dependence of antigen redistribution was similar whether the Cd-MT was injected subcutaneously or intravenously, showing that the phenomenon was not limited by the rate of Cd-MT resorption and transport to the kidney. Rather, the rate-limiting step may be
endocytosis and intracellular processing of filtered Cd-MT by proximal tubules and/or the rate of antigen internalization via endocytosis. However, when the effects of Cd in the form of Cd-MT and CdCl₂ were compared, the inorganic Cd caused the initial step of granulation, but no subsequent intracellular antigen redistribution was apparent. This finding agrees with previous observations in rats and mice, in which inorganic Cd may bind Cd and prevent immediate toxicity. Nevertheless, the key player in nephrotoxicity seems to be Cd; rats treated with ZnMT showed no functional and structural damage to proximal tubules (61). In (sub)chronic Cd nephrotoxicity, after endocytosis and lysosomal degradation of filtered Cd-MT (20), the liberated Cd may stimulate the production of MT in proximal tubule cells (23, 55, 59), which may bind Cd and prevent immediate toxicity. This may be why, in experimental animals treated with small doses of CdCl₂ for weeks or months, Cd in the kidney cortex has to reach a “critical” concentration of ~200 μg/g wet mass to overcome the binding capacity of newly synthesized MT before the unbound Cd becomes nephrotoxic (24, 53, 55). However, the data in this report (although representing more acute conditions) show that tissue Cd in the renal cortex reached only ~40 μg/g wet mass 3 h after Cd-MT injection, and yet there was a marked internalization of BBM transporters. At shorter time points, when the processes of granulation (1 h) and endocytosis (2 h) had already begun, the tissue Cd concentration was probably even lower. This indicates that Cd may act as a nephrotoxin immediately after its release from lysosomes, at much lower intracellular levels than previously thought, supporting the conclusions of other studies (16, 31).

Although the intracellular events that trigger Cd-MT-induced endocytosis of BBM transporters are not known, our data suggest that depolymerization of microtubules may be an important event in this process. Fragmentation and loss of microtubules in proximal tubule cells were already extensive 1 h after Cd-MT injection, while the internalization of megalin was still at the initial granulation stage at this time point. This suggests that depolymerization of microtubules may precede the process of endocytosis of BBM transporters. As the loss of microtubules became more extensive over the next 2–3 h, the internalization of megalin was also more extensive. At 6 and 12 h after Cd-MT injection, patches of microtubules in a state of apparent repolymerization were detected. At the same time, the significantly increased amount of α-tubulin in tissue homogenates, which in its nonpolymerized state cannot be readily visualized by immunocytochemistry, indicates that Cd-MT either stimulated synthesis or inhibited degradation of α-tubulin, or both. The patches of repolymerized but disorganized microtubules may indicate the start of regenerative processes in proximal tubule cells that take place 6–12 h after a single Cd-MT injection. These rapid regenerative processes may be specific for acute Cd-MT toxicity, as in this study, but may be slower in animals treated with multiple, short-interval Cd-MT treatments, e.g., in conditions that may be more relevant for chronic Cd intoxication (23, 38, 47, 70). However, we recently reported a similar limited loss and/or derangement of microtubules, actin filaments, and villin in proximal convoluted tubules of rats treated with inorganic Cd for 2 wk (56), suggesting that similar phenomena occur in (sub)chronic Cd nephrotoxicity. In the present report, other cytoskeletal components were not examined, but our data indicate that the effect of Cd on microtubules might be an early event that may trigger other cellular responses, beginning with inhibition of the vesicle-mediated recycling of BBM transporters. The process continues with a loss of BBM components and microvilli, leading ultimately to more general structural and functional damage. However, a direct connection between depolymerization of microtubules and internalization of BBM transporters in Cd-MT-treated rats, as suggested by our data, remains to be established in future experiments.
As shown in our previous studies (6, 13, 26, 56), colchicine-induced loss of microtubules in proximal convoluted tubule cells led to a similar internalization of several BBM transporters, including megalin, V-ATPase, AQP1, and NHE3. However, only NHE3 was also relocated into the BLM after microtubule disruption (56). These findings were reproduced in this study after Cd-MT injection. Normally an apical protein, NHE3 was detected in the BLM 6 and 12 h after Cd-MT treatment, suggesting that Cd-MT had a colchicine-like effect on vesicle trafficking, resulting in a partial loss of cell polarity. This supports our recent hypothesis that the intracellular recycling of NHE3 in proximal tubules may include microtubule-independent targeting of newly synthesized NHE3 to the BLM and subsequent microtubule-dependent internalization and transcytosis to the apical membrane (56). After depolymerization of microtubules by colchicine (56) or Cd-MT (this study), NHE3 may slowly accumulate in the BLM due to disrupted internalization and deficient transcytosis. This would explain why the accumulation of NHE3 in the BLM was more pronounced at 12 h than at 6 h after Cd-MT treatment. Our gold-labeling data also support this contention. Whereas the BBM in the affected tubules was nearly depleted of megalin 12 h after Cd-MT exposure, the amount of gold-labeled NHE3 in this membrane domain was actually greater than in control tissues. This indicates the presence of (at least) two pools of BBM proteins that respond differently to Cd-MT exposure and microtubule disruption. Some proteins, such as megalin (and probably the V-ATPase), are rapidly internalized under these conditions and are removed from the BBM in a time-dependent manner (indicated by the decreasing density of the corresponding protein bands in immunoblots). Other proteins, such as NHE3 (and probably AQP1), are internalized more slowly and may even become more concentrated in the BBM as the total amount of BBM decreases over time. By Western blotting, significant differences in the apical abundance of these antigens that would correspond more closely to the immunocytochemical observations were probably

Fig. 7. Immunolocalization of α-tubulin in proximal convoluted tubules of control rats (A) and of rats treated with Cd-MT 1 (B), 2 (C), 4 (D), 6 (E), and 12 h (F) earlier. In the tubules of control rats, α-tubulin was arranged in apicobasally oriented bundles of microtubules. One hour after Cd-MT-treatment (B), the staining was partially lost, and the remaining microtubules were heavily fragmented. The loss of microtubules was stronger 2 h (C) and nearly complete 4 h (D) after Cd-MT treatment. Patches of staining (arrowheads), probably indicating sites of partial repolymerization of microtubules, began to reappear at many sites at 6 h (E) and more extensively 12 h (F) after Cd-MT injection. Bar = 20 μm.
partially masked by a contribution from the BBM of proximal tubules in which Cd-MT-induced protein redistribution was less marked or absent.

How could Cd-MT cause depolymerization of microtubules in the proximal convoluted tubule? Being highly reactive with thiol groups, Cd released from Cd-MT could directly chelate essential SH groups in highly reactive with thiol groups, Cd released from Cd-MT could directly chelate essential SH groups in tubulin and block polymerization of tubulin monomers (25, 40, 45, 46). In addition, in a model of experimental Cd-MT nephrotoxicity identical to that used in the present study, a strongly reduced uptake of 45Ca into tubulin and block polymerization of tubulin monomers could be demonstrated (51). At least some of these processes may also take place in proximal tubule cells and elevate the intracellular concentration of Ca2+, which is a powerful inducer of microtubule disassembly (25, 49, 51).

Because microtubules are involved in vesicle trafficking and maintaining epithelial cell polarity (14), depolymerization of microtubules may have deleterious consequences with regard to the structure and function of proximal tubule cells. Together with a functional V-ATPase in various organelles, microtubules are required for the continuous recycling of various domain-specific membrane proteins (14). Recent data have demonstrated that vesicle acidification is necessary for the selective recruitment of various vesicle coat proteins, including Arf6, ARNO, and β-coat protein from the cell cytosol (Ref. 42 and references therein), a process that may play an important role in intracellular vesicle trafficking and receptor-mediated endocytosis. We have shown previously that inorganic Cd inhibits V-ATPase activity and impairs acidification in various organelles of the vacuolar system in proximal tubule cells (28). This process may also contribute to the inhibitory effect of Cd on the recycling of BBM transporters. With time, the diminished expression of many apical proteins in the proximal tubule cell BBM (Refs. 28, 30, and 53 and the present study) and the impaired endocytosis of proteins from the ultrafiltrate (28, 30) may contribute to the loss of proximal tubule structure and function that is associated with Cd-induced nephrotoxicity.

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